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## Inducing protective immune responses against the sporozoite and liver stages of *Plasmodium*<sup>1</sup>

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## Inducing protective immune responses against the sporozoite and liver stages of *Plasmodium*<sup>1</sup>

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### 1. Summary

Work on vaccines against the pre-erythrocytic stages of the *Plasmodium* life cycle is based on the observation that immunization with irradiated sporozoites (IRR SPZ) is protective. Antibodies against several SPZ surface proteins can prevent SPZ from effectively invading hepatocytes; antibodies and cytolytic-T lymphocytes directed against at least 3 parasite proteins expressed in infected hepatocytes can kill infected hepatocytes; and cytokines can activate infected hepatocytes to kill the intracellular parasite. Work is in progress to identify additional pre-erythrocytic parasite targets and to develop methods for optimally inducing protective immunity against SPZ and infected hepatocytes. The goal is to construct a vaccine that protects by inducing antibody and cellular immune responses against multiple parasite proteins.

### 2. Foundation for pre-erythrocytic malaria vaccine development: the irradiated sporozoite model

Immunization of mice, monkeys and humans with IRR SPZ protects against SPZ-induced malaria [1–9]. In humans this immunity is stage specific. It does not protect against challenge with infected erythrocytes; the protective immunity must be directed against the SPZ or infected hepatocyte. The immunity is not strain specific [2,6], and lasts for at least 9 months [10].

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<sup>1</sup> The opinions and assertions herein are those of the authors and are not to be construed as official or as reflecting the views of the US Navy or the naval service at large.

### 3. Preventing sporozoites from effectively invading hepatocytes

#### 3.1. Rationale

Passive transfer of monoclonal antibodies (mAbs) against the repeat regions of the *P. berghei* (Pb) [11] and *P. yoelii* (Py) [12] circumsporozoite proteins (PbCSP and PyCSP) protects mice against SPZ challenge, and passive transfer of a mAb against the *P. vivax* CSP (PvCSP) protects monkeys [13]. These mAbs also block SPZ invasion into hepatocytes in vitro.

#### 3.2. Data from animal model systems

Over the past 7 years a series of vaccines designed to produce antibodies to the PbCSP and PyCSP have been tested in mice [14–17]. Progress has been steady and up to 100% protection has now been achieved in the highly infectious Py system with a vaccine that only contains repeats from the PyCSP as malaria material (Wang, R. et al., submitted).

#### 3.3. Human trials

More than 15 vaccines designed to induce antibodies against the repeat regions of the *P. falciparum* (PfCSP) and PvCSP have been evaluated in volunteers [summarized in 18]. These vaccines have varied in the composition of B-cell epitopes, T-cell epitopes, and delivery systems. The B epitopes have been synthetic peptides and purified recombinant proteins, the T epitopes have come from large carrier proteins including tetanus toxoid [19] and *Pseudomonas aeruginosa* toxin A [20], non-*Plasmodium* recombinant fusion proteins such as the non-structural protein of influenza A [21–23], and the flanking regions of the PfCSP [24]. The

delivery systems/adjuvants have included aluminum hydroxide [19,25]; monophosphoryl lipid A (MPL), cell-wall skeleton of mycobacteria and squalane [22,26]; AIOH<sub>3</sub> and MPL; the combination of AIOH<sub>3</sub>, MPL and liposomes [23]; and hepatitis B particles with AIOH<sub>3</sub> and MPL [27]. The combination of AIOH<sub>3</sub>, MPL, and liposomes has been most immunogenic. Currently about 20–25% of volunteers can be protected against SPZ challenge by these vaccines. Future plans include testing multiple antigen peptide vaccines based on the repeat region of the PfCSP. In addition a vaccine that does not include the repeat region of the PfCSP, only the flanking regions, is undergoing testing [28].

### *3.4. Identification of other targets for preventing effective SPZ invasion of hepatocytes*

Work is in progress to identify other targets on the SPZ; antibodies against different proteins would hopefully have additive effects. It would be ideal to identify ligands on the SPZ for hepatocyte receptors and to induce antibodies that prevent receptor-ligand interaction. The region II domain of the CSP has been identified as such a region [29–31].

## **4. Attacking the infected hepatocyte**

### *4.1. IRR SPZ vaccine-induced protective immunity dependent on CD8<sup>+</sup> T cells*

Although antibodies against the CSP are completely protective, the protection induced by IRR SPZ is probably primarily mediated by T cells directed against infected hepatocytes. Purified T cells from mice immunized with IRR SPZ adoptively transfer protection to naive mice [14], and depletion of CD8<sup>+</sup> T cells from some strains of fully immune mice eliminates protection [32,33].

### *4.2. Association between the presence of HLA-Bw53 (class I), and protection against severe malaria*

In Gambia HLA-Bw53 was found in 16.9% of cases of severe malaria, in 25.4% of controls with mild malaria, and in 25% of adults without malaria [34]. The relative risk of severe malaria among individuals with HLA-Bw53 compared with those without this allele was 0.59. HLA-Bw53 is found in 15–40% of the population of sub-Saharan Africa, but is found in less than 1% of Caucasians and Orientals; like the sickle-cell trait, it may have been selected because it protects against severe malaria. The presence of HLA-Bw53 is not as protective as the HbS carrier state; only 1.2% of pa-

tients with severe malaria in the Gambia study had HbS, while 12.9% of controls with mild malaria carried HbS (relative risk: 0.08, corresponding to > 90% protection against severe malaria). Nonetheless the decreased association of HLA-Bw53 with severe malaria suggests that naturally acquired CTL against *Plasmodium* sp. proteins protect against severe malaria. The logical targets for CTL are infected hepatocytes. Most deaths occur in children younger than 5 years of age, and these children are not protected against developing malaria infections. Thus, such data raise the possibility that under natural conditions of exposure, CTL against infected hepatocytes reduce mortality by reducing the burden of infection, not by preventing infection. This is consistent with reports that insecticide-impregnated bednets have a more profound effect on mortality than on incidence of malaria [35].

### *4.3. CD8<sup>+</sup> cytotoxic-T lymphocytes against the CSP are protective in adoptive transfer*

CD8<sup>+</sup> CTL against a short sequence on the PyCSP eliminate Py-infected hepatocytes from culture in an antigen specific, MHC restricted manner [36]. These CTL as well as analogous CTL from the PbCSP adoptively transfer protection in vivo [37–39]. In the case of Py CTL clones this has been shown to occur even if CTL are transferred 3 h after SPZ inoculation at a time when the SPZ have invaded hepatocytes, and that the presence on the surface of the CTL clone of CD44, an adhesion protein, is required for protective activity [40]. This suggests that in addition to epitope specificity, correct homing is required for protective activity of CTL.

### *4.4. Immunization with CSP vaccines induces CD8<sup>+</sup> T-cell dependent partial protection in rodent malaria*

With the knowledge that CTL against a single epitope on the Pb and Py CSPs could completely prevent SPZ-induced malaria, scientists have tried to induce such CTL using subunit vaccines. In the Pb system CTL were induced by immunizing with recombinant *S. typhimurium* or vaccinia expressing PbCSP. Immunization with the recombinant *S. typhimurium* produced 60–70% protection that was dependent on CD8<sup>+</sup> T cells [41]. The recombinant vaccinia induced CTL, but did not protect [42]. In the Py model immunization with recombinant *S. typhimurium* [43], vaccinia [44] and pseudorabies virus [45] expressing the PyCSP induced excellent immune responses, but no protection. However, recently it was shown that a primary injection of recombinant influenza virus expressing the PyCS CTL epitope, followed by a booster of a recombinant vac-

cinia expressing the entire PyCSP gave 60% protection [46]. Immunization with a recombinant P815 cell expressing PyCSP induced up to 75% protection that was dependent on CD8<sup>+</sup> T cells [47]. An exciting recent development has been the demonstration that immunization with PyCSP plasmid DNA induces high levels of antibodies and CTL and protects more than 50% of mice [48].

#### *4.5. Development of vaccines to induce CTL against the PfCSP in humans*

It was first established that humans immunized with IRR Pf SPZ had cytolytic activity against the PfCSP. This cytolytic activity was dependent on CD8<sup>+</sup> T cells, antigen-specific and genetically restricted, and was directed against target cells expressing the entire PfCSP and those pulsed with a synthetic peptide comprising amino acids 368–390 of the 7G8 PfCSP [49]. Among the first 4 volunteers tested, 3 had such activity. When these volunteers were challenged, 3 of the 4 were protected. The 1 volunteer who was not protected was shown to have CTL against PfCSP, and 1 volunteer who was protected was not shown to have such CTL [9,49]. CTL against the PfCSP have also been identified in Kenyans [50], Australians with previous exposure to malaria [51], and among HLA B35 Gambians [52]. Among the Gambians a 9-amino-acid peptide, amino acids 368–376 of PfCSP, labeled target cells for killing. One human volunteer immunized with recombinant *S. typhi* expressing PfCSP had antigen-specific, CD8<sup>+</sup> T cell-dependent cytolytic activity against the PfCSP [53]. Work is in progress to determine if immunization with recombinant PfCSP protein with several adjuvants, including alum, MPL, and liposomes induces CTL against PfCSP in humans.

#### *4.6. Identification of SPZ surface protein 2 (SSP2) as a target of vaccine induced CD8<sup>+</sup> protective CTL*

In analyzing the protective immunity induced by the IRR SPZ vaccine a number of investigators came to the conclusion that this immunity must be directed against multiple parasite proteins. One approach to identifying such proteins was to immunize mice with IRR SPZ, and produce mAbs. Using this approach the gene encoding a 140 kDa Py SPZ protein was characterized [54–56]. Mice immunized with IRR Py SPZ were shown to produce CTL against this protein [47], and adoptive transfer of a CTL clone against this protein called PySSP2 protected mice against sporozoite challenge, even if transferred 3 h after SPZ inoculation when all SPZ were in hepatocytes [57]. Immunization of mice with recombinant P815 mastocytoma cells expressing a

1.5 kb fragment of PySSP2 protected 50% of mice [47]. The protection was dependent on CD8<sup>+</sup> T cells. The Pf homolog of PySSP2 is the previously described thrombospondin related anonymous protein (TRAP) [58,59], and efforts are underway to develop vaccines designed to induce protective immune responses against PfSSP2.

#### *4.7. Immunization with CSP plus SSP2 gives additive protection*

Knowing that immunization with P815 cells expressing PyCSP or PySSP2 each produced 50–75% protection, mice were immunized with the combination and 100% were protected [47]. This protection was eliminated by in vivo depletion of CD8<sup>+</sup> T cells.

#### *4.8. CD4<sup>+</sup> CTL against the CSP mediate protective immunity*

CD4<sup>+</sup> T cells that recognize epitopes within amino acids 59–79 of the PyCSP eliminate infected hepatocytes from culture and adoptively transfer protection [60,61]. Immunization of mice with the analogous peptide from the PbCSP induced partial protection [62]. Furthermore, a CD4<sup>+</sup> CTL clone that recognizes a peptide including amino acids 337–346 of the 7GS PfCSP has been derived from a human immunized with IRR Pf SPZ [63]. Thus, there is now considerable interest in inducing protective CD4<sup>+</sup> CTL against the PfCSP.

#### *4.9. Identification of additional targets of protective immune responses on infected hepatocytes*

There are several perspectives among investigators working to discover important liver stage antigens. Since immunization with IRR SPZ provides potent protective immunity, one approach is to limit the investigation to antigens that are present at the latest stage of development of IRR SPZ in hepatocytes, and against which antibody or cell responses are induced by immunization with IRR SPZ. Both the CSP and SSP2, which are present in SPZ and infected hepatocytes, were discovered using this approach. The second approach is to look for any antigen expressed in infected hepatocytes, regardless of its role in IRR SPZ-induced protective immunity, and assess its capacity to induce protective immune responses. A Pf liver stage-specific protein, liver stage antigen-1 (LSA-1), and its proposed Pb homolog, LSA-2, a Pb liver stage-specific protein, Pb liver 1 (Pbl-1); and a 17 kDa Py liver and blood-stage protein, PyLB17, have all been discovered using the second approach. LSA-1 was identified by screening a genomic library using sera from individuals who had

spent long periods of time in Africa taking chemoprophylaxis [64]. Pb11 was identified using a mAb generated by immunizing mice with Pb-infected hepatoma cells 1 [65]. The mAb, anti-Pb11, is specific to the exo-erythrocytic stage and does not react with SPZ or blood stages. Passive transfer of the mAb did not protect against SPZ challenge, but did reduce parasite density; the mAb did not affect the growth of liver stages in vitro [66]. PyLB17 was discovered by immunizing mice with hepatocytes from mice infected in vivo with large numbers of Py SPZ 43 h previously and producing a mAb that does not recognize SPZ, but recognizes infected hepatocytes and erythrocytes. This mAb eliminates Py- but not Pb-infected hepatocytes from culture and reduces parasitemia after SPZ and blood-stage challenge [Charoenvit et al., submitted]. Work is in progress to clone the gene encoding this protein, and to identify its Pf homolog.

In addition, the Pf major merozoite surface protein-1 (MSP-1) is expressed in late liver schizonts [67], and PfExp-1 [68] is expressed throughout the liver cycle. These or other 'blood'-stage antigens that are also 'liver'-stage antigens may be the targets of protective antibody and T-cell responses when expressed in infected hepatocytes.

#### *4.10. Identification of PflSA-1 as a potential target of CTL*

To follow up the demonstration of an association between HLA-Bw53 and lack of severe malaria, peptides eluted from HLA-Bw53 were sequenced and studied in CTL assays in Gambia [52]. Among HLA Bw53 positive individuals, 3 of 6 volunteers in a village with high transmission of malaria, and 1 of 9 volunteers in a village with lower transmission had HLA-Bw53-restricted, peptide-specific, CD8<sup>+</sup> T cell-dependent cytolytic activity against a peptide from an LSA-1 peptide referred to as 1s6 (KPIVQYDNF). This epitope was shown to be invariant in 9 *P. falciparum* isolates. These findings suggest that CTL against this LSA-1 peptide may be involved in the partial protection against severe malaria associated with HLA-Bw53.

#### *4.11. Role of IFN $\gamma$ and other cytokines in protective immunity against infected hepatocytes*

The mechanisms by which CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes actually eliminate infected hepatocytes from culture and protect in vivo are not established; however, cytokines probably play a major role. The data regarding IFN $\gamma$  is the strongest. IFN $\gamma$  partially protects mice and monkeys against Pb and *P. cynomolgi*

respectively [69,70], and in vitro treatment of infected hepatocytes with IFN $\gamma$  eliminates Pf from culture [71]. Treatment with anti-IFN $\gamma$  of A/J mice immunized with Pb IRR SPZ eliminates protective immunity [32]. This is not the case with BALB/c mice [72]. Adoptive transfer of a CD8<sup>+</sup> T-cell clone against the PyCSP that endogenously produces large quantities of IFN $\gamma$  protects against Py, and this protective immunity is eliminated by in vivo treatment of mice with anti-IFN $\gamma$  [39].

Analysis of secretion patterns of certain CD4<sup>+</sup> T-cell clones suggests that other cytokines are also involved in protection [73]. IL-1 and IL-6 inhibit intrahepatic development of human and murine parasites [71,74]. Tumor necrosis factor (TNF) inhibited development of Pb in vitro in a hepatoma cell line [75], but was not effective alone in primary cultures of Py-infected hepatocytes [76]. However, in co-culture of hepatocytes and non-parenchymal cells, TNF induced parasite inhibition by IL-6 release [76,77]. Recent work with IL-12 also suggests a role for this cytokine in protection [Sedegah et al., submitted].

The mechanism by which cytokines kill infected hepatocytes is not well established. Recent reports indicate that IFN $\gamma$  and perhaps other cytokines induce infected hepatocytes to produce L-arginine-derived nitrogen oxides that are toxic to the intracellular parasite [77,78].

### **5. The future: inducing multiple immune responses against multiple targets**

Vaccines against the pre-erythrocytic stages of the parasite are designed to completely protect against malaria or, if used in combination with an erythrocytic stage vaccine, to substantially reduce the number of inoculated SPZ that develop to mature liver-stage schizonts and release infective merozoites. We know that this can be achieved because immunization of humans with IRR Pf SPZ is protective. It seems logical that the protective immunity induced by the attenuated 'whole organism' vaccine is directed against multiple targets and mediated by multiple immune mechanisms. We know that mAbs against the CSP repeats expressed on circulating SPZ, CD8<sup>+</sup> CTL against a single epitope on the CSP, CD4<sup>+</sup> CTL against a single epitope on the CSP, and CD8<sup>+</sup> CTL against a single epitope on SSP2, all presumably expressed in infected hepatocytes, can each completely protect against SPZ-induced malaria in the absence of other parasite-specific immune responses. Furthermore, antibodies against PyLB17 eliminate infected hepatocytes from culture, presumably by recognizing this protein expressed in infected hepatocytes. Thus 5 discrete targets on the SPZ and infected

hepatocytes and at least 3 different immune responses can be completely protective.

It is likely that the IRR SPZ vaccine induces protective immunity against additional targets, and work is in progress to identify them. Nonetheless it seems that a coherent strategy would be to try to produce vaccines for humans that induce these varied responses. Just as immunization with IRR Pf SPZ seems to protect all individuals immunized, regardless of the strain of Pf with which they are challenged, such multivalent vaccines should overcome the problems of restriction of responses in specific individuals and variation of target epitopes.

During the past decade there have been enormous advances in our understanding of the mechanisms and targets of IRR SPZ-induced protective immunity. Current efforts focus on development of methods for optimal vaccine construction and delivery so as to maximize required immune responses against multiple targets. Once effective vaccines are developed, the question of expense of production and delivery will also have to be addressed if such vaccines are ever to be available to the people who need them most. Nonetheless there is now great hope that we will one day have vaccines that protect against malaria by attacking the parasite at multiple stages in its pre-erythrocytic cycle, and that such vaccines will be combined with vaccines that attack the asexual and sexual stages of the parasite life cycle.

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